

Docosahexanoic Acid-Induced Coronary Arterial Dilation: Actions of 17S-Hydroxy Docosahexanoic Acid on K⁺ Channel Activity

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ABSTRACT

Despite extensive studies, the mechanisms mediating the cardiovascular actions of ω -3 polyunsaturated fatty acids has not yet been fully understood. The present study determined the possible actions of an endothelium-derived lipoxygenase product of docosahexanoic acid (DHA), 17S-hydroxy DHA (17S-HDHA), in bovine coronary arteries. High-performance liquid chromatography (LC) analysis demonstrated that bovine coronary arterial endothelial cells can metabolize DHA via lipoxygenases, and one of the major products was confirmed to be 17S-HDHA by LC-tandem mass spectrometry. In isolated perfused small bovine coronary arteries, 17S-HDHA (10^{-9} to 10^{-5} M) caused a concentration-dependent dilation with a maximum dilator response of $87.8 \pm 2.5\%$, which is much more potent than the dilator response of its precursor, DHA. Moreover,

17S-HDHA-induced vasodilations were significantly blocked by iberiotoxin, a large conductance Ca^{2+} -activated K⁺ (BK_{Ca}) channel blocker, but not altered by an ATP-sensitive K⁺ channel blocker, glibenclamide. In patch-clamp whole-cell recording, 17S-HDHA markedly increased K⁺ currents in coronary arterial smooth muscle cells. In the inside-out mode, but not in the cell-attached mode, 17S-HDHA dramatically increased the BK_{Ca} channel activity, which was substantially blocked by iberiotoxin. Collectively, our findings indicate that 17S-HDHA, an endothelium-derived DHA product via lipoxygenase, activates BK_{Ca} channels in coronary arterial smooth muscle cells, leading to coronary vasodilation, which may represent an important mechanism mediating the beneficial actions of DHA in coronary circulation.

Introduction

Numerous epidemiological studies, clinical trials, and animal experiments have demonstrated that fish oils, primarily ω -3 polyunsaturated fatty acids (PUFAs), protect against several types of cardiovascular diseases such as myocardial infarction, arrhythmia, atherosclerosis, stroke, or hypertension (Rapp et al., 1991; McLennan et al., 1996; Nageswari et al., 1999; Kang and Leaf, 2000; Abeywardena and Head, 2001; De Caterina and Zampolli, 2001; Jeerakathil and Wolf, 2001; Leaf et al., 2003; Holub and Holub, 2004; Harrison and Abhyankar, 2005). Two well known ω -3 PUFAs present in fish oil are docosahexanoic acid (DHA) and eicosapentaenoic

acid (Connor et al., 1993). Studies have indicated that DHA may be a major active component in fish oil conferring cardiovascular protection (Horrocks and Yeo, 1999; Nordøy et al., 2001; Hirafuji et al., 2003). In animal experiments, DHA was found more effective than eicosapentaenoic acid in retarding the development of hypertension in spontaneously hypertensive rats and inhibiting thromboxane-like vasoconstrictor responses in the aorta from these rats (McLennan et al., 1996). However, it remains poorly understood how DHA exerts its beneficial action on the cardiovascular system, but several possible mechanisms have been suggested, such as reduction of plasma triglycerides, inhibition of platelet function, enhancement of cardiac excitability, and anti-inflammation (McLennan et al., 1996; Salem et al., 2001; Simopoulos, 2002).

DHA has been found to be metabolized via cyclooxygenase, lipoxygenase, and P450 metabolic pathways, which generate a series of 17R or 17S monohydroxy, dihydroxy, and trihy-

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ABBREVIATIONS: PUFA, polyunsaturated fatty acid; DHA, docosahexanoic acid; 17S-HDHA, 17S-hydroxy DHA; EC, endothelial cell; SMC, smooth muscle cell; BK_{Ca}, large conductance Ca^{2+} -activated K⁺; CDC, cinnamyl-3, 4-dihydroxyl-cyanocinnamate; LC, liquid chromatography; HPLC, high-performance LC; MS, mass spectrometry; ID, internal diameter; U46619, (Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid; EET, epoxyeicosa-5,8,11-trienoic acid; DHET, dihydroxyeicosatrienoic acid.

droxy DHA and various epoxides (Hong et al., 2003). Some of these DHA products possess potent bioactivity, in particular, being active as anti-inflammatory and immune-regulatory compounds (Hong et al., 2003). Inflammation or microinflammation plays important roles in the development of atherosclerosis, ischemic reperfusion injury, and cardiac or vascular remodeling. In this regard, the anti-inflammatory or immune-regulatory effects of DHA and its products have been suggested to contribute to the beneficial actions of ω -3 PUFAs or fish oil on the cardiovascular system (Simopoulos, 2002; Holub and Holub, 2004). However, many classic anti-inflammatory drugs such as commonly used indole and aryl-propionic acid derivatives do not have similar cardiovascular protective actions to that observed in DHA treatments. This suggests that some other mechanisms are involved in the action of DHA or ω -3 PUFAs on the cardiovascular system in addition to their anti-inflammatory effects. In this regard, previous studies demonstrated that a ω -3 PUFA diet enhanced endothelium-dependent vasodilator response in coronary arteries (Shimokawa and Vanhoutte, 1989; Fleischhauer et al., 1993). Therefore, DHA may exert its beneficial action through an endothelium-dependent mechanism in coronary circulation.

The present study hypothesized that 17S-HDHA, a lipoxygenase product, mediates the endothelium-dependent vasodilator action of DHA in small coronary arteries. To test this hypothesis, we first separated and analyzed the lipoxygenase metabolites of DHA produced in coronary arteries and endothelial cells (ECs). Then, we tested the ability and potency of 17S-HDHA to produce vasodilator response in isolated perfused coronary arteries. We further determined whether vasodilator response to 17S-HDHA is associated with the activation of K^+ channels by using the patch-clamp technique. Our data indicate that 17S-HDHA is a much more potent vasodilator than DHA, and the vasodilator action of 17S-HDHA is associated with the activation of large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels in coronary arterial smooth muscle cells (SMCs).

Materials and Methods

Video Microscopy of Arterial Reactivity. Isolated pressurized small coronary artery preparation was used to study the vasomotor response to DHA and its metabolites as we described previously (Geiger et al., 2000). In brief, the internal diameter (ID) of these arteries was measured with a microscopic video recording system composed of a stereomicroscope (Leica MZ8; Leica, Wetzlar, Germany), a charge-coupled device camera (KP-MI AU; Hitachi, Tokyo, Japan), a video monitor (VM-1220U; Hitachi), a video measuring apparatus (VIA-170; Boeckeler Instruments, Tucson, AZ), and a video printer (UP890 MD; Sony, Tokyo, Japan). The arterial images were also recorded continuously with a videocassette recorder (M-674; Toshiba, Tokyo, Japan). Before testing any compounds, the cannulated artery was equilibrated for 1 to 1.5 h and then precontracted to 40 to 60% of their resting diameter with a thromboxane A_2 analog, (Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo [2.2.1]heptan-6-yl]hept-5-enoic acid (U46619) (2–20 nM). Once steady-state contraction was obtained, cumulative concentration response to 17S-HDHA (10^{-9} to 10^{-5} M; kindly provided by C. S. Serhan, Harvard Medical School, Boston, MA) or DHA (10^{-9} to 10^{-5} M; Cayman Chemical, Ann Arbor, MI) were determined by measuring changes in the ID. The vasodilator response was expressed as percentage of relaxation relative to U46619-induced precontraction as calculated in the following formula: percentage of relaxation = $(ID_{\text{dilator}} - ID_{\text{U46619}})/ID_{\text{basal}}$

– ID_{U46619} , where ID_{basal} means the arterial ID under resting condition; ID_{U46619} indicates the ID of U46619-precontracted arteries; and ID_{dilator} means the arterial ID obtained during treatment of U46619-precontracted arteries with compounds tested. Between pharmacological interventions, the arteries were washed three times with physiological salt solution and allowed to equilibrate in drug-free physiological salt solution for 20 to 30 min. The arteries were treated with corresponding inhibitors for 20 min and then concentration-response curves to 17S-HDHA were redetermined. To prepare endothelium-denuded small coronary arteries, the rings of arteries were passed through its lumen by a segment of hair. The endothelium was removed by gently rotating the hair (Ge et al., 2003).

Extraction and Analysis of DHA Metabolites. DHA metabolites were analyzed from the reaction mixtures of arterial or cell homogenates with DHA. The homogenates of small coronary arteries were prepared as described previously (Geiger et al., 2000). The reactions were performed by incubating 200 μ g of protein of homogenates prepared from the arteries, ECs, or SMCs, with DHA (100 μ M, 15 min at 37°C) with a final volume of 200 μ l. The incubation was stopped upon addition of 2 ml of methanol, which was prechilled to -40°C and placed at -40°C for 30 min. Then, the reaction mixtures were mixed with an internal standard of 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (1 ng/ μ l), centrifuged at 3000 rpm for 15 min at 4°C, and then acidified to pH 3.5 with HCl. The extraction manifold was set up using a vacuum apparatus and Extract-Clean/RC500 mg C18 Cartridges (Alltech Associates, Deerfield, IL) (one cartridge per sample) and washed with methanol and distilled H_2O . The samples were run through the cartridges followed by washing of distilled H_2O and hexane. The fraction was then collected after the addition of 8 ml of methyl formate through the respective cartridges. The samples containing lipid extracts were dried, redissolved in methanol, and prepared for analysis. The identity of DHA metabolite in the extracts was confirmed by using HPLC coupled with a photodiode-array detector and a tandem mass spectrometer (LC-PDA-MS-MS; Thermo Fisher Scientific, Waltham, MA).

In HPLC analysis, C^{14} -labeled DHA (1 μ Ci) was used for each reaction. The extracts obtained as described above were dissolved in acetonitrile and run on a reversed-phase HPLC system on a nucleosil C_{18} column (5 μ m, 4.6×250 mm; Phenomenex, Torrance, CA). The solvent program consisted of solvent A that was distilled water and solvent B that consisted of acetonitrile containing 1% glacial acetic acid. A linear gradient from 50% solvent B to 100% solvent B in solvent A over 40 min was used at a flow rate of 1 ml/min. The discharge was collected in 0.2-ml fractions and mixed with scintillation fluid, and then the radioactivity was measured by liquid scintillation spectrometry to obtain a profile of radioactive metabolites.

Western Blot Analysis. Forty micrograms of proteins (homogenates) prepared from coronary arteries, ECs, and SMCs were boiled, separated by 12% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The blots were blocked by 5% nonfat dry milk solution and incubated with polyclonal antibody against 15-lipoxygenase (kindly provided by W. B. Campbell, Medical College of Wisconsin, Milwaukee, WI) overnight at 4°C. After washing three times with TBS-T, the blots were incubated for 1 h with 1:4000 horseradish peroxidase-labeled goat anti-rabbit IgG and developed using the enhanced chemiluminescence detection system (ECL; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

Patch-Clamp Recording. K^+ currents were recorded using the patch-clamp technique according to the procedures described previously (Hamill et al., 1981; Gebremedhin et al., 1992; Li and Campbell, 1997). Freshly isolated coronary arterial SMCs were prepared for patch-clamp studies as described previously (Li and Campbell, 1997). The bath solutions used for whole-cell recording mode contained 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, 10 mM glucose, pH 7.4, and the pipette solution contained 145 mM L-glutamic acid, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM

EGTA (K⁺), 1 mM Na₂ATP, 0.5 mM NaGTP, and 100 nM ionized Ca²⁺, pH 7.2. The bath solutions used for single-channel recordings in the cell-attached mode contained 145 mM KCl, 1 mM MgCl₂, 1 mM EGTA (K⁺), 10 mM HEPES, and 100 nM ionized Ca²⁺, pH 7.4, and the pipette solution contained 145 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4. The bath solutions used in the inside-out recording mode contained 145 mM KCl, 1 mM MgCl₂, 5 mM HEPES, and ionized 100 nM Ca²⁺, pH 7.2, and the pipette solution contained 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4. All the solutions were stored at 4°C until use.

Patch pipettes were made from borosilicate glass capillary tubing that were pulled with a two-stage micropipette puller (PC-87; Sutter Instrument Company, Novato, CA) and heat-polished with a microforge (MF-90; Narishige, Tokyo, Japan). The pipettes had tip resistances of 2 to 5 MΩ for whole-cell recordings when filled with pipette solution. SMCs were placed in a 1-ml perfusion chamber mounted on the stage of a Nikon inverted microscope. A high-resistance seal (5–10 GΩ) was formed between the pipette tip and the cell membrane by applying light suction. The activity of K⁺ channels in the membrane spanning the pipette tip was recorded. These measurements represented the cell-attached mode. To establish a whole-cell recording configuration, a large pulse of suction was used to rupture the membrane within the pipette, creating a direct continuity between the pipette solution and cytoplasmic contents of the cell. Inside-out membrane patches were excised by lifting the pipette membrane complex to the air/solution interface. Channel currents were recorded using voltage-clamping mode of a List EPC-7 patch-clamp amplifier (List Biological Laboratories, Inc., Campbell, CA). For a whole-cell pulse protocol, the patched cell was stimulated by voltage pulses from –60 to +60 mV in steps of 10 mV. Cell capacitance (C_m) was estimated in each cell by integrating capacitive currents generated by 10-mV hyperpolarizing pulses after electronic cancellation of the pipette-patch capacitance (pF). Peak K⁺ current amplitudes were expressed in picoamperes per picofarad (pA/pF) to normalize for differences in cell membrane area between isolated bovine coronary arterial SMCs. Here we kept cells with capacitance as 17.2 ± 0.7 pF. For the cell-attached or inside-out recording configurations, 3-min recordings were obtained at a membrane potential of –40 mV before or after treatments. Single-channel conductance (pS) was calculated as amplitude (pA) divided by holding potential (mV). Data were digitized, acquired, and stored using a Micron (Boise, ID)

Pentium III computer and Axon pClamp software (8.1) (Molecular Devices, Sunnyvale, CA).

The effects of 17S-HDHA on the BK_{Ca} channel activity were first determined with the whole-cell recording mode. After a whole-cell patch was established, control recordings were obtained, and then the bath solution was changed to contain 17S-HDHA and a second pulse recording at each concentration was obtained. To further determine the effects of these 17S-HDHA-induced activation of the K⁺ channels, patch-clamp recordings were performed in the cell-attached and inside-out patch mode. A 3-min control recording was obtained at a membrane potential of –40 mV. The bath solution was then exchanged as described above in recording channel activity by whole-cell patch mode, and then a second successive 3-min recording was obtained at each concentration. In another group of cells, ibertoxin (100 nM) was added into the pipette solution and the same recording protocol was repeated.

Average channel activity (NP_o) in patches was determined from the following:

$$NP_o = (\sum_{j=1}^N t_{ij})/T \quad (1)$$

where N is the maximal number of channels observed in conditions producing high levels of P_o , T is the duration of the recording, and t_j is the time with $j = 1, 2 \dots N$ channels opening.

Statistics. Data are presented as mean ± S.E.M.; n represents the number of independent experiments. The significant differences in mean values between and within multiple groups were examined using an analysis of variance for repeated measures followed by a Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Metabolism of DHA in Coronary Arteries, ECs, and SMCs. We first determined whether small coronary arteries produce hydroxy metabolites of DHA via the lipoxygenase pathway. In this regard, bovine coronary arterial homogenates were incubated with ¹⁴C-DHA in the presence and absence of the lipoxygenase inhibitor, cinnamyl-3,4-dihydroxyl-cyanocinnamate (CDC) (10 μM, both 15- and 5-lipoxygenase are inhibited), and the DHA metabolites in the reac-

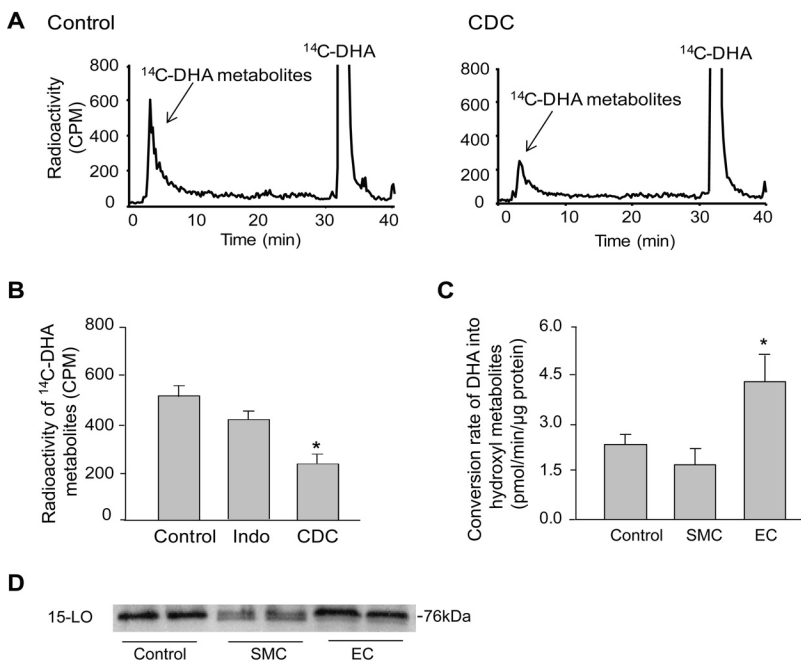


Fig. 1. Metabolism of DHA in coronary arteries. ¹⁴C-DHA was incubated with homogenates of bovine coronary arteries for 15 min at 37°C in the presence or absence of indicated inhibitors. Then DHA and its metabolites in the reaction mixtures were extracted and applied for HPLC analysis. A, typical HPLC chromatograms depicting the metabolism of ¹⁴C-DHA with (left) or without addition of the lipoxygenase inhibitor CDC (10 μM; right) in coronary arterial tissue homogenates. Metabolites of ¹⁴C-DHA were eluted at a peak of 2.5 min, and ¹⁴C-DHA was eluted at a peak of 33 min. B, summarized data showing radioactivity of ¹⁴C-DHA metabolites in the reaction mixtures in the absence or presence of the cyclooxygenase inhibitor indomethacin (Indo, 100 μM) or CDC (10 μM) ($n = 4$). C, summarized data showing the conversion rates of DHA to its metabolites in homogenates of coronary arterial tissue (Control), ECs, and SMCs ($n = 5$). D, representative Western blot gel document showing 15-lipoxygenase (15-LO) expression in homogenates of coronary arterial tissue (Control) and their cells (SMC and EC). *, $P < 0.05$, significant difference compared with the values from control samples.

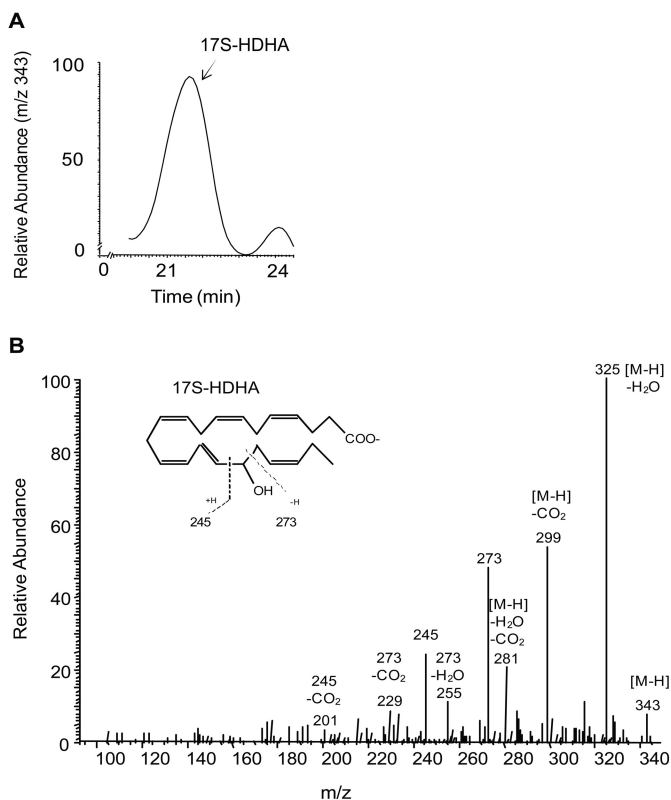


Fig. 2. LC/MS analysis of DHA metabolites. A, typical LC chromatogram of 17S-HDHA from LC/MS analysis showing that 17S-HDHA had a peak at a retention time of 21 min. B, typical MS spectrum of lipidomic MS/MS analysis of DHA metabolites isolated from bovine coronary arteries. Inset, chemical structure of 17S-HDHA as reported previously (Hong et al., 2003). 17S-HDHA was identified on the basis of the diagnostic MS-MS ions at m/z of 201 (245-CO₂), 229 (273-CO₂), 245, 255 (273-H₂O), 273, 281 (M-H-H₂O-CO₂), 299 (M-H-CO₂), 325 (M-H-H₂O), and 343 (M-H).

tion mixture were extracted followed by separation and analysis by HPLC. Figure 1A presents typical HPLC chromatograms depicting the hydroxy metabolites of ¹⁴C-DHA with a radioactive peak at 2.5 min. Inhibition of lipoxygenase by CDC markedly reduced the peak of DHA metabolites. As summarized in Fig. 1B, the conversion of DHA into hydroxy metabolites was significantly blocked by CDC but not by the cyclooxygenase inhibitor, indomethacin (100 μ M). Similar to indomethacin, P450 monooxygenase inhibitors, miconazole (10 μ M) or 17-octadecynoic acid (1 μ M), had no effect on such metabolism of DHA (data not shown). In addition, the hydroxy metabolites of DHA were detected in homogenates from coronary arterial ECs and SMCs (Fig. 1C). The conversion rate of DHA into hydroxy metabolites was 3-fold higher in ECs than in SMCs (Fig. 1C). 15-Lipoxygenase has been shown to hydrolyze the 17S position of DHA to form 17S-HDHA (Hong et al., 2003). As indicated by Western blot analysis, 15-lipoxygenase has higher abundance in ECs than in SMCs (Fig. 1D), suggesting that the high conversion rate of DHA into hydroxy metabolites in ECs is associated with the higher abundance of 15-lipoxygenase in these cells.

LC/MS Spectrometric Analysis of DHA Metabolites.

Figure 2A shows a typical LC chromatogram of 17S-HDHA obtained from LC/MS analysis, which demonstrates that 17S-HDHA had the largest peak at a retention time of 21 min. Figure 2B presents a MS spectrum and structure of

17S-HDHA. The identity of 17S-HDHA was confirmed by ions at m/z of 201 (245-CO₂), 229 (273-CO₂), 245, 255 (273-H₂O), 273, 281 (M-H-H₂O-CO₂), 299 (M-H-CO₂), 325 (M-H-H₂O), and 343, which was then further confirmed by MS/MS analysis. Our HPLC and LC/MS analyses further identified that 17S-HDHA is the primary form of hydroxy metabolites; however, other 17S series hydroxy metabolites of DHA, including di- and tri-HDHA, were also detected (data not shown). 5-Lipoxygenase metabolizes 17S-HDHA into di- and tri-HDHA (Hong et al., 2003). Thus, our data indicate that endogenous 15-lipoxygenase and 5-lipoxygenase in bovine coronary arteries are able to metabolize DHA into 17S series HDHA.

Vasodilator Responses to 17S-HDHA and DHA in Small Coronary Arteries. We next tested whether DHA or 17S-HDHA cause coronary vasodilation. As shown in Fig. 3A, in isolated precontracted small bovine coronary arteries, DHA induced vasodilator response at a relatively high concentration (10^{-5} M) with a maximal dilation of $56.9 \pm 6.2\%$. Compared with its precursor DHA, 17S-HDHA induced a much stronger vasodilator response with a maximal dilation of $87.8 \pm 2.5\%$ at a concentration of 10^{-5} M. Moreover, 17S-HDHA at 10^{-7} M induced an obvious dilator response ($42.9 \pm 4.9\%$), whereas DHA had only a trivial dilator response ($11.9 \pm 2.5\%$) at the same dose. To quantitate the potency of dilator responses for 17S-HDHA and DHA, we calculated the concentration required to induce 50% dilation

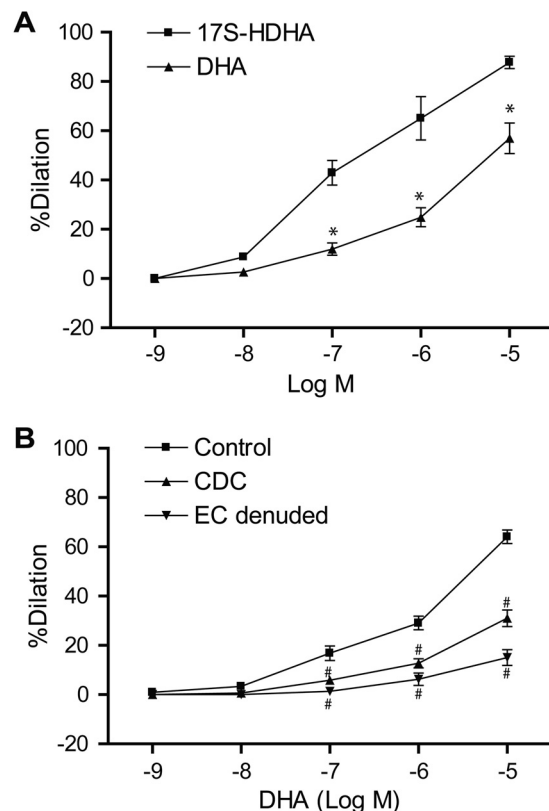


Fig. 3. A, DHA and 17S-HDHA induce vasodilation of bovine small coronary arteries. *, $P < 0.05$, significant difference compared with the values from the arteries treated with DHA ($n = 6$). B, effects of CDC (10 μ M) and endothelium removal (EC denuded) on DHA-induced vasodilation in bovine small coronary arteries. #, $P < 0.05$, significant difference compared with the values from the endothelium intact arteries treated with DHA ($n = 6$).

(D₅₀) and found that the 17S-HDHA (D₅₀ = 2.0×10^{-7} M) was 31-fold more potent than DHA (D₅₀ = 6.3×10^{-6} M). As shown in Fig. 3B, inhibition of lipoxygenase by CDC significantly reduced DHA-induced vasodilation. Furthermore, removal of the endothelium abolished DHA-induced vasodilation in these coronary arteries.

Involvement of K⁺ Channels in 17S-HDHA-Induced Vasodilation. We further examined whether 17S-HDHA-induced vasodilation is associated with K⁺ channel activation. In this regard, we first investigated the effect of the combination of several K⁺ channel inhibitors on 17S-HDHA-induced coronary vasodilation. It was found that treatment of bovine coronary arteries with a cocktail of inhibitors including iberiotoxin (100 nM), 4-aminopyridine (5 mM), and glibenclamide (100 μ M) markedly attenuated 17S-HDHA-induced vasodilation with maximum dilation of $24.7 \pm 3.4\%$ at 10^{-5} M ($n = 4$; data not shown). Then, different K⁺ channel blockers were used separately to determine the specific subtypes of K⁺ channels that are involved in the vasodilator response of 17S-HDHA. As shown in Fig. 4A, the BK_{Ca} channel inhibitor, iberiotoxin (100 nM) significantly attenuated 17S-HDHA-induced maximal vasodilation by 46%. 4-Aminopyridine (5 mM), an inhibitor of voltage-gated K⁺ channels, also significantly attenuated 17S-HDHA-induced maximal vasodilation by 50% (Fig. 4B). However, the ATP-sensitive K⁺ channel blocker glibenclamide (100 μ M) had no effect (Fig. 4C).

Effects of 17S-HDHA on K⁺ Channel Activity Recorded by a Whole-Cell Patch-Clamp Configuration. To further demonstrate the involvement of BK_{Ca} channels in 17S-HDHA-induced vasodilation, patch-clamp experiments were performed to examine whether this DHA metabolite alters BK_{Ca} channel currents. Bovine coronary smooth muscle cells were freshly dissociated and transferred to the patch-clamp chamber with a bath solution for whole-cell current recording. Figure 5A shows the recordings of outward K⁺ channel current from SMCs by whole-cell configuration. In this cell, 17S-HDHA (10^{-6} M) increased K⁺ current by 130%. Outward K⁺ channel currents (pA) were normalized to the averaged cell capacitance (17.2 ± 0.7 pF). The summarized current density-voltage relationship is presented in Fig. 5B. In contrast, DHA at the same concentration did not increase the K⁺ current recorded by whole-cell configuration. The currents at different ramp voltage were very similar in bovine arterial SMCs with and without administration of 10^{-6} M DHA (Fig. 6A). As summarized in Fig. 6B, administration of DHA (10^{-6} M) had no effect on the current density-voltage relationship in these cells.

Effects of 17S-HDHA on Single BK_{Ca} Channel Activity in Cell-Attached Patches. To further examine the mechanism of 17S-HDHA-induced activation of BK_{Ca} channels, we performed single-channel recordings in the cell-attached mode in SMCs. Figure 7A shows representative recordings of single BK_{Ca} channels in the cell-attached mode under control condition and treated with 10^{-6} M 17S-HDHA. The BK_{Ca} channel conductance was 250.7 pS, and the opening amplitude was 10 pA with clamped voltage at -40 mV. 17S-HDHA (10^{-6} M) slightly increased the channel open probability (NP_o) of these BK_{Ca} channels. Figure 7B summarizes the effect of 17S-HDHA on the NP_o of BK_{Ca} channels. Under control conditions, the NP_o of this

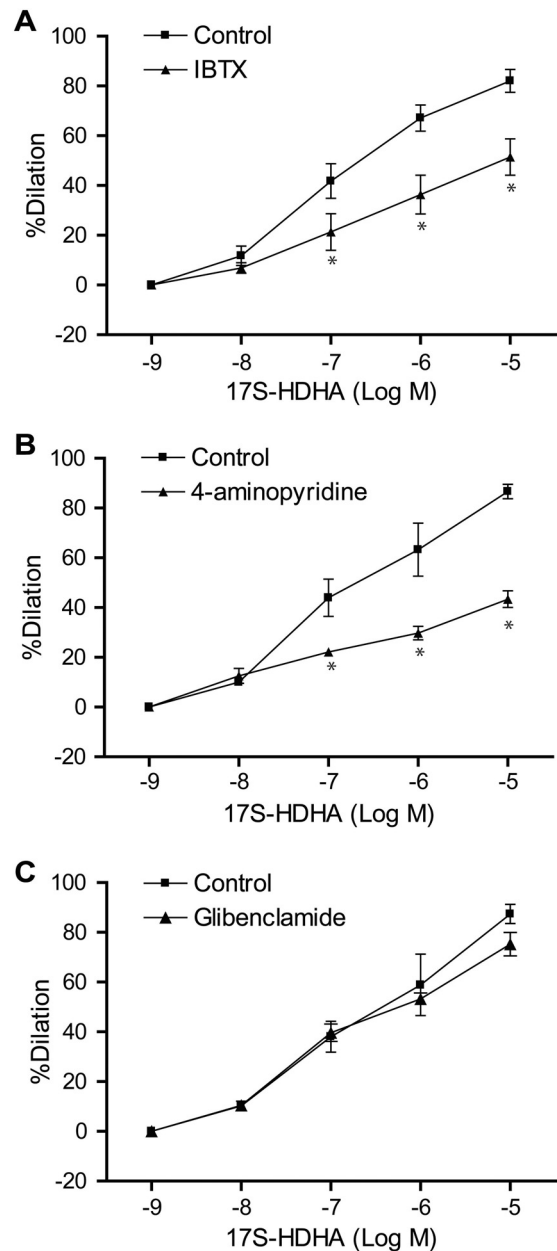


Fig. 4. Effects of the BK_{Ca} channel blocker iberiotoxin (IBTX, 100 nM) (A), the K_v channel blocker 4-aminopyridine (5 mM) (B), and the K_{ATP} channel blocker glibenclamide (100 μ M) (C) on 17S-HDHA-induced vasodilation in bovine small coronary arteries ($n \geq 4$). *, $P < 0.05$, significant difference compared with the values from the control arteries that are treated with 17S-HDHA only.

K⁺ channel was 0.0067 ± 0.0029 . 17S-HDHA (10^{-8} to 10^{-6} M) caused a slight, but not significant, increase in the NP_o of BK_{Ca} channels.

Effects of 17S-HDHA on Single BK_{Ca} Channel Activity in Inside-Out Patches. Figure 8A shows representative recordings of BK_{Ca} channels in an inside-out patch of SMCs in the absence of cytosolic ATP and GTP. Administration of 17S-HDHA (10^{-7} M) into the bath solution, the cytosolic face of the patch, markedly increased BK_{Ca} channel openings. As summarized in Fig. 8B, 17S-HDHA (10^{-9} to 10^{-7} M) significantly increased the NP_o of these K⁺ channels in a concentration-dependent manner. This increase in the NP_o of BK_{Ca} channels by 17S-HDHA (10^{-7} M)

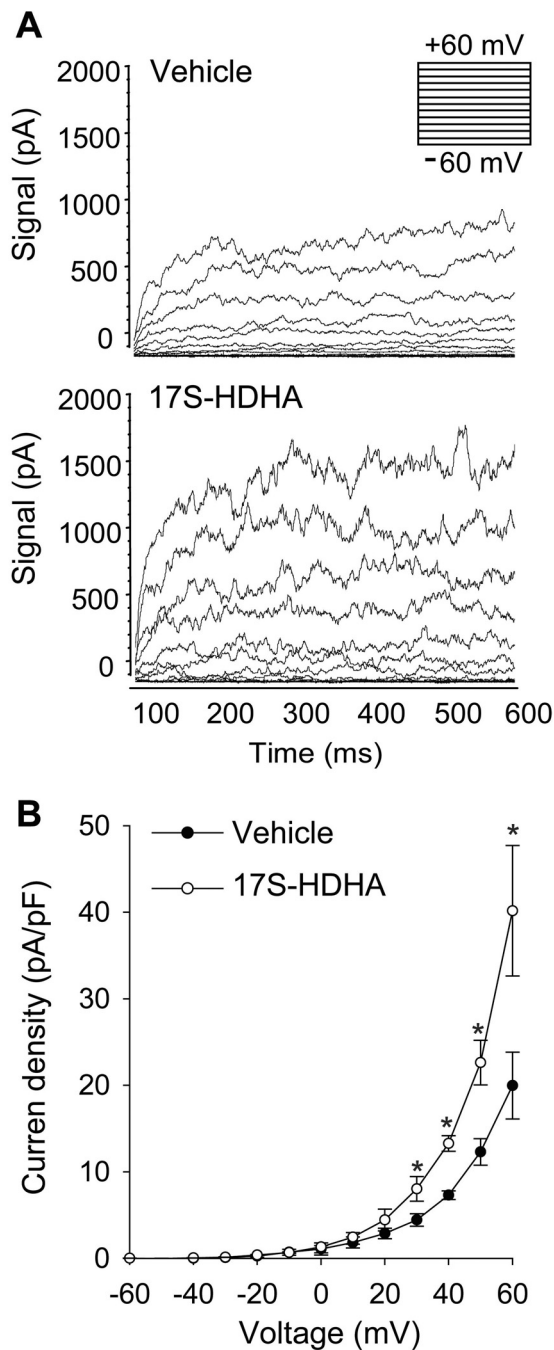


Fig. 5. Effect of 17S-HDHA on whole-cell K^+ channel currents in bovine coronary SMCs. A, representative recordings of outward K^+ currents in one SMC under control conditions or treated with 10^{-6} M 17S-HDHA. B, summarized K^+ current densities activated by voltage showing that 17S-HDHA (10^{-6} M) induced increases in K^+ currents. *, $P < 0.05$, significant difference compared with the values from vehicle-treated bovine coronary SMCs.

was almost completely eliminated by iberiotoxin (100 nM) in the patch pipette.

Discussion

The present study reported that 17S-HDHA, a lipoxygenase product of DHA, is produced in coronary arteries and possesses much more potent vasodilator action than DHA in isolated, perfused, and pressurized small coronary arteries.

The potent vasodilator effect of this DHA metabolite is associated with the activation of the BK_{Ca} channel in coronary arterial SMCs. These findings support the view that DHA may exert its cardioprotective effects through the regulation of vasomotor responses via its metabolites in coronary circulation.

In previous studies, exogenous 15-lipoxygenase was reported to hydrolyze the 17S position of DHA to form 17R- or 17S-HDHA (monohydroxy), which can be further converted by 5-lipoxygenase into 17S series hydroxy DHA including dihydroxy or trihydroxy DHA (Hong et al., 2003). By HPLC and LC-MS-MS analysis, we found that DHA in coronary arterial homogenates was converted mainly into 17S series hydroxy DHA, including 17S-HDHA (monohydroxy), dihydroxy, and trihydroxy HDHA and that 17S-HDHA is the

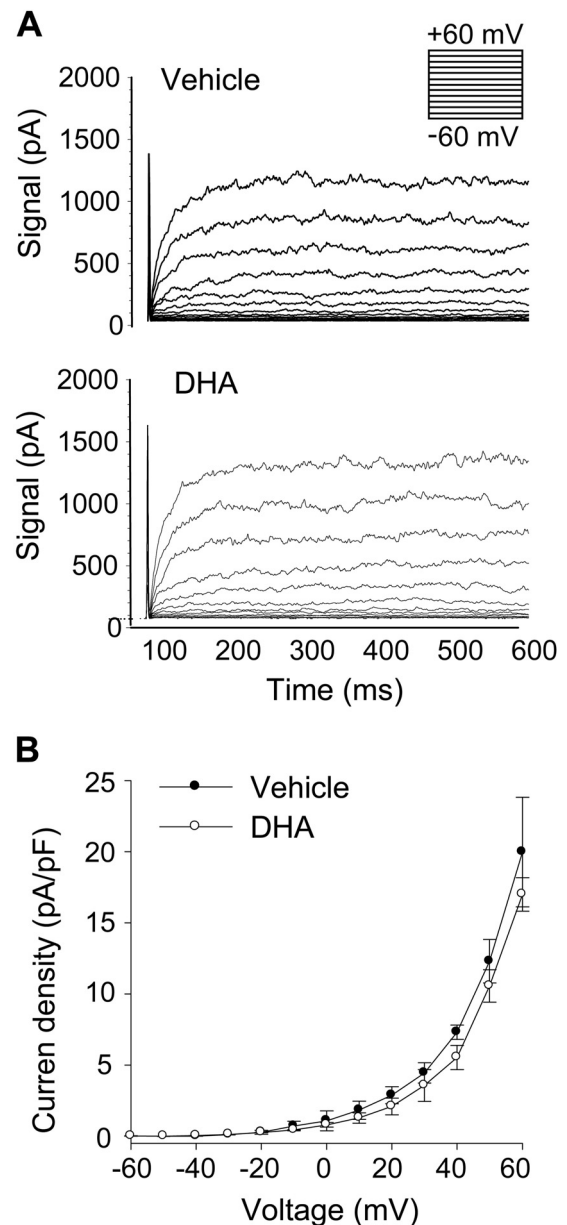


Fig. 6. Effect of DHA on whole-cell K^+ channel currents in bovine coronary SMCs. A, representative recordings of outward K^+ currents in one bovine coronary SMC under control conditions or treated with 10^{-6} M DHA. B, summarized K^+ current densities activated by voltage showing that DHA (10^{-6} M) did not increase K^+ currents ($n = 6$).

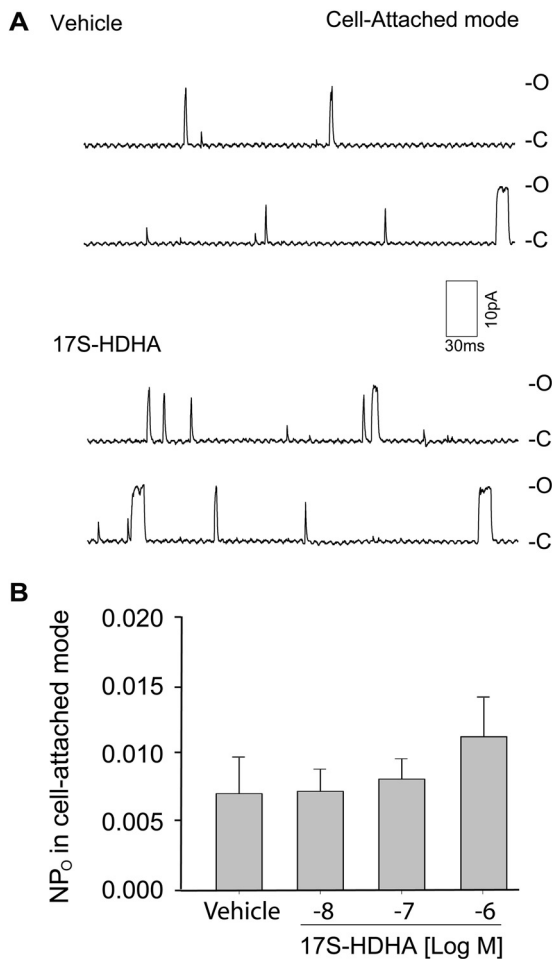


Fig. 7. Effects of 17S-HDHA on single BK_{Ca} channel activity in the cell-attached mode. **A**, representative recordings of the BK_{Ca} channels in bovine coronary SMCs under control conditions or treated with 17S-HDHA (10^{-6} M) at a voltage of -40 mV (o, open; c, closed). **B**, summarized data showing the effects of 17S-HDHA (10^{-8} to 10^{-6} M) on NP_o of BK_{Ca} channels ($n = 6$).

major form of this series hydroxy DHA produced by coronary arteries using DHA as substrate. It was also demonstrated that the metabolism of DHA was blocked by the lipoxygenase inhibitor CDC, but not by the cyclooxygenase inhibitor indomethacin, suggesting that 15- and 5-lipoxygenase synergistically convert DHA into its hydroxy metabolites in small bovine coronary arteries. Consistently, we showed that inhibition of lipoxygenases by CDC markedly attenuated DHA-induced dilation in small bovine coronary arteries, suggesting the dilator action of DHA is mediated primarily through its hydroxy metabolites. The expression level of 15-lipoxygenase and the conversion rate of DHA into 17S-HDHA are much higher in ECs than in SMCs. We further demonstrated that endothelial removal abolished DHA-induced dilation in small bovine coronary arteries. Therefore, these data indicate that DHA is metabolized mainly into 17S-HDHA in the endothelium rather than smooth muscle of coronary arteries.

To our knowledge, the present study for the first time demonstrated that 17S-HDHA possesses much more potent vasodilator effect than its precursor, DHA, in small bovine coronary arteries. In this regard, several previous studies tested the potency of DHA in dilating porcine or rat coronary arterioles, human coronary arteries, or rat aortic rings (En-

gler and Engler, 2000; Ye et al., 2002; Wu et al., 2007). However, those authors found that DHA significantly induced dilation only when high concentrations of DHA were used (typically more than 10^{-5} M). We found here that 17S-HDHA induced potent dilation in small bovine coronary arteries at a low concentration of 10^{-7} M, whereas DHA only weakly dilated these arteries at the same concentration. The precise mechanism responsible for this differential vasodilator action between 17S-HDHA and DHA is unknown. The chemical structure of DHA shares similarity with arachidonic acid, which is metabolized into potent vasodilators 14,15-EET and 14,15-DHET by cytochrome P450 in coronary arteries (Campbell et al., 2002). Because these arachidonic acid metabolites are structurally related with 17S-HDHA, it is plausible that they share some common structural elements required for their vasodilator activity. Falck et al., 2003 have suggested that the 8,9-double bond and 14,15-epoxide/14,15-dihydroxy group are essential elements for the vasodilator activity of 14,15-EET/14,15-DHET. The 8,9-double bond may be involved in p-p bonding with aromatic amino acids of potential receptors (Gauthier et al., 2002; Falck

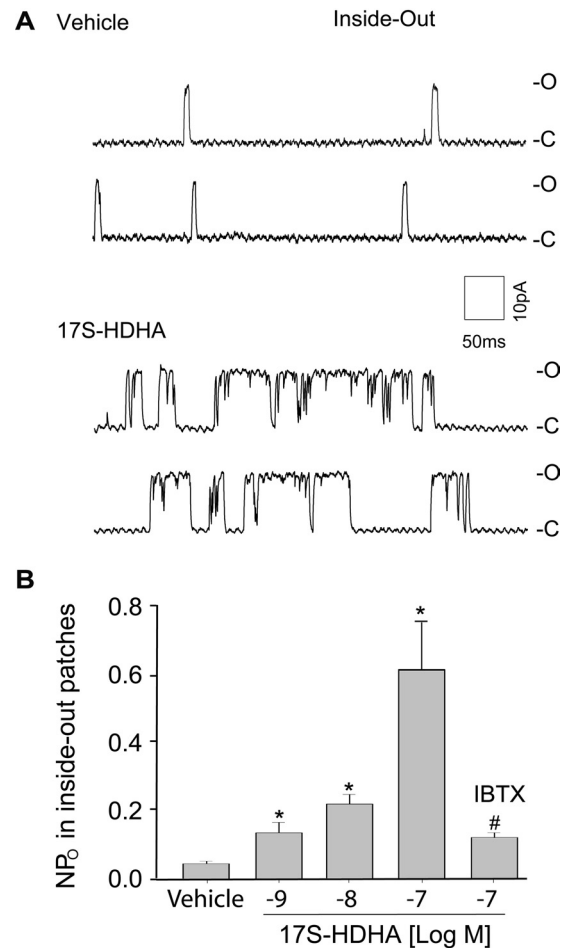


Fig. 8. Effects of 17S-HDHA on single BK_{Ca} channel activity in inside-out patches. **A**, representative recordings of the BK_{Ca} channels in bovine coronary SMCs under control conditions or treated with 17S-HDHA (10^{-7} M) at a voltage of -40 mV (o, open; c, closed). **B**, summarized data showing the effects of 17S-HDHA on NP_o of the BK_{Ca} channels in the absence or presence of iberiotoxin (IBTX, 100 nM) in the patch pipette ($n = 6$). *, $P < 0.05$, significant difference compared with the values from vehicle-treated patches; #, $P < 0.05$, significant difference compared with the values from patches treated with 10^{-7} M 17S-HDHA.

et al., 2003), whereas the oxygen atoms of the epoxide or dihydroxy group may act as acceptors for hydrogen bonding with receptors of 14,15-EET/14,15-DHET. Based on these findings, we hypothesized that the 10,11-double bond and 16,17-dihydroxy group are essential structural requirements for the potent vasodilator action of the hydroxy metabolites of DHA. Hydroxylation at carbon 17 of DHA molecule may increase hydrogen bonding with the potential receptor and thereby enhance the vasodilator activity. This may explain why 17S-HDHA exerts a much more potent vasodilator effect than DHA.

Regulation of smooth muscle membrane potential through alteration in K^+ channel activity is a major mechanism of vasodilation and vasoconstriction under both physiological and pathophysiological conditions (Yokoshiki et al., 1998; Toro et al., 2002; Jackson, 2005). Activation of K^+ channels results in hyperpolarization, closure of voltage-dependent Ca^{2+} channels, and vasodilation, whereas inhibition of K^+ channels has the opposite effects. Several different types of K^+ channels including BK_{Ca} channel, K_v channel, and K_{ATP} channel, are present in bovine coronary SMCs and involved in the regulation of vascular tone or vasomotor responses of coronary arteries (Yokoshiki et al., 1998; Toro et al., 2002; Jackson, 2005). In the present study, we found that both the BK_{Ca} blocker, iberiotoxin, and the K_v channel blocker, 4-aminopyridine, significantly attenuated 17S-HDHA-induced vasodilation, whereas the K_{ATP} channel blocker, glibenclamide, had no effect. These data suggest that the activation of BK_{Ca} and K_v channels may contribute to 17S-HDHA-induced vasodilation in coronary arteries.

To further determine the electrophysiological mechanism-mediating actions of 17S-HDHA, we performed patch-clamp experiments with a focus on the BK_{Ca} channel in the membrane of coronary arterial SMCs. In the whole-cell recording mode, 17S-HDHA (10^{-6} M) markedly increased K^+ currents in these SMCs. In contrast, DHA (10^{-6} M) had no effect on the K^+ currents, suggesting that DHA at such doses does not activate BK_{Ca} channels in bovine coronary SMCs. It seems that these results are inconsistent with a study demonstrating that DHA activates BK_{Ca} channels in rat coronary arterial SMCs (Lai et al., 2009). In rat SMCs, however, the increase in BK_{Ca} currents was observed only when high concentrations of DHA (2.0×10^{-5} M to 8.0×10^{-5} M) were used (Lai et al., 2009). It is possible that 17S-HDHA and a high concentration of DHA may activate BK_{Ca} channels via distinct mechanisms. 17S-HDHA-induced activation of BK_{Ca} channels seems to be specific to the structure of 17S-HDHA as shown in EET studies (Campbell et al., 2002; Gauthier et al., 2002; Falck et al., 2003). 17S-HDHA may directly interact with and change the conformation of BK_{Ca} channels, leading to increased channel activity. As reported by Thid et al. (2007), DHA at high concentrations (more than 6.0×10^{-5} M) dramatically changed the viscoelastic properties of the phosphocholine-supported lipid bilayers. Thus, DHA at high concentrations is more likely to exert its effects as a nonspecific lipid compound that modifies biophysical properties of membranes, which results in the alteration of BK_{Ca} channel activities.

Previous studies demonstrated that the direct action of $G_{S\alpha}$ on the BK_{Ca} channels mediates the effects of 11,12-EET, an important endothelium-derived hyperpolarization factor (Brown and Birnbaumer, 1990; McDonald et al., 1994; Li and

Campbell, 1997). This membrane-delimited action of $G_{S\alpha}$ is a ubiquitous mechanism for regulating K^+ and Ca^{2+} channels and requires the presence of GTP (Brown and Birnbaumer, 1990; McDonald et al., 1994). However, our data suggest that a direct activation of G proteins is not involved in 17S-HDHA-induced BK_{Ca} channel activation. First, in the cell-attached mode, 17S-HDHA had no significant effects on BK_{Ca} channel activity, suggesting that 17S-HDHA does not activate BK_{Ca} channel through intracellular coupling mechanisms. This result suggests a membrane-delimited action of 17S-HDHA on BK_{Ca} channel activity. In excised inside-out patches, 17S-HDHA markedly increased BK_{Ca} channel activity when it was added to the cytoplasmic side of membranes even in the absence of GTP or ATP. This suggests that the activation of BK_{Ca} channels by 17S-HDHA does not require any intracellular soluble components, including GTP and ATP. These data rule out the possibility of a G protein-coupled mechanism in mediation of 17S-HDHA action. It seems that a membrane-delimited, but G protein-independent, mechanism mediates 17S-HDHA-induced activation of BK_{Ca} channels.

It should be noted that 17S-HDHA also induced a small residual dilation in the presence of a combination of K^+ channel inhibitors (i.e., iberiotoxin, 4-aminopyridine, and glibenclamide) in bovine small coronary arteries with maximum dilation of $24.7 \pm 3.4\%$ at 10^{-5} M (Fig. 3B). This residual dilator response may be attributed to the dilator effects of 17S-HDHA metabolites. It has been shown that 17S-HDHA can be further converted by 5-lipoxygenase into 17S series hydroxy DHA including dihydroxy or trihydroxy DHA (Hong et al., 2003). It is possible that these 17S-HDHA metabolites (converted by lipoxygenases in the intact endothelium) dilate coronary arteries via K^+ channel-independent mechanisms. Indeed, we found that 17S trihydroxy DHA is also a potent vasodilator in bovine small coronary arteries with a maximal dilation of $59.1 \pm 5.5\%$ at 10^{-7} M (unpublished data). However, the mechanisms responsible for the dilator effect of 17S trihydroxy DHA are unknown and need to be further investigated in future studies.

In summary, the present study demonstrated that 17S-HDHA is a novel and potent vasodilator in coronary arteries. 17S-HDHA activates BK_{Ca} channels in SMCs from these arteries via a membrane-delimited mechanism by direct action of 17S-HDHA on channel proteins or their regulatory proteins. Because 17S-HDHA possesses more potent vasodilator effect than DHA itself, this DHA metabolite may serve as an endothelium-derived hyperpolarization factor to regulate vascular tone in coronary circulation and to at least partially mediate the beneficial actions of DHA or ω -3 PUFAs in the prevention and treatment of cardiovascular diseases, in particular coronary arterial diseases.

Authorship Contributions

Participated in research design: X. Li, P.-L. Li, and Zhang.

Conducted experiments: X. Li, Hong, and Zhang.

Performed data analysis: X. Li, Hong, P.-L. Li, and Zhang.

Wrote or contributed to the writing of the manuscript: X. Li, P.-L. Li, and Zhang.

References

- Abeywardena MY and Head RJ (2001) Longchain n-3 polyunsaturated fatty acids and blood vessel function. *Cardiovasc Res* 52:361–371.

- Brown AM and Birnbaumer L (1990) Ionic channels and their regulation by G protein subunits. *Annu Rev Physiol* **52**:197–213.
- Campbell WB, Deeter C, Gauthier KM, Ingraham RH, Falck JR, and Li PL (2002) 14,15-Dihydroxyeicosatrienoic acid relaxes bovine coronary arteries by activation of K(Ca) channels. *Am J Physiol Heart Circ Physiol* **282**:H1656–H1664.
- Connor WE, DeFrancesco CA, and Connor SL (1993) N-3 fatty acids from fish oil. Effects on plasma lipoproteins and hypertriglyceridemic patients. *Ann NY Acad Sci* **683**:16–34.
- De Caterina R and Zampolli A (2001) n-3 fatty acids: antiatherosclerotic effects. *Lipids* **36**:S69–S78.
- Engler MB and Engler MM (2000) Docosahexaenoic acid-induced vasorelaxation in hypertensive rats: mechanisms of action. *Biol Res Nurs* **2**:85–95.
- Falck JR, Krishna UM, Reddy YK, Kumar PS, Reddy KM, Hittner SB, Deeter C, Sharma KK, Gauthier KM, and Campbell WB (2003) Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation. *Am J Physiol Heart Circ Physiol* **284**:H337–H349.
- Fleischhauer FJ, Yan WD, and Fischell TA (1993) Fish oil improves endothelium-dependent coronary vasodilation in heart transplant recipients. *J Am Coll Cardiol* **21**:982–989.
- Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR, and Campbell WB (2002) 14,15-Epoxyeicosa-5(Z)-enoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. *Circ Res* **90**:1028–1036.
- Ge ZD, Zhang DX, Chen YF, Yi FX, Zou AP, Campbell WB, and Li PL (2003) Cyclic ADP-ribose contributes to contraction and Ca²⁺ release by M1 muscarinic receptor activation in coronary arterial smooth muscle. *J Vasc Res* **40**:28–36.
- Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M, and Harder DR (1992) Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am J Physiol Heart Circ Physiol* **263**:H519–H525.
- Geiger J, Zou AP, Campbell WB, and Li PL (2000) Inhibition of cADP-ribose formation produces vasodilation in bovine coronary arteries. *Hypertension* **35**:397–402.
- Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* **391**:85–100.
- Harrison N and Abhyankar B (2005) The mechanism of action of omega-3 fatty acids in secondary prevention post-myocardial infarction. *Curr Med Res Opin* **21**:95–100.
- Hirafuji M, Machida T, Hamaue N, and Minami M (2003) Cardiovascular protective effects of n-3 polyunsaturated fatty acids with special emphasis on docosahexaenoic acid. *J Pharmacol Sci* **92**:308–316.
- Holub DJ and Holub BJ (2004) Omega-3 fatty acids from fish oils and cardiovascular disease. *Mol Cell Biochem* **263**:217–225.
- Hong S, Gronert K, Devchand PR, Moussignac RL, and Serhan CN (2003) Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J Biol Chem* **278**:14677–14687.
- Horrocks LA and Yeo YK (1999) Health benefits of docosahexaenoic acid (DHA). *Pharmacol Res* **40**:211–225.
- Jackson WF (2005) Potassium channels in the peripheral microcirculation. *Microcirculation* **12**:113–127.
- Jeerakathil TJ and Wolf PA (2001) Prevention of strokes. *Curr Atheroscler Rep* **3**:321–327.
- Kang JX and Leaf A (2000) Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. *Am J Clin Nutr* **71**:202S–207S.
- Lai LH, Wang RX, Jiang WP, Yang XJ, Song JP, Li XR, and Tao G (2009) Effects of docosahexaenoic acid on large-conductance Ca²⁺-activated K⁺ channels and voltage-dependent K⁺ channels in rat coronary artery smooth muscle cells. *Acta Pharmacol Sin* **30**:314–320.
- Leaf A, Xiao YF, Kang JX, and Billman GE (2003) Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids. *Pharmacol Ther* **98**:355–377.
- Li PL and Campbell WB (1997) Epoxyeicosatrienoic acids activate K⁺ channels in coronary smooth muscle through a guanine nucleotide binding protein. *Circ Res* **80**:877–884.
- McDonald TF, Pelzer S, Trautwein W, and Pelzer DJ (1994) Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol Rev* **74**:365–507.
- McLennan P, Howe P, Abeywardena M, Muggli R, Raederstorff D, Mano M, Rayner T, and Head R (1996) The cardiovascular protective role of docosahexaenoic acid. *Eur J Pharmacol* **300**:83–89.
- Nageswari K, Banerjee R, and Menon VP (1999) Effect of saturated, omega-3 and omega-6 polyunsaturated fatty acids on myocardial infarction. *J Nutr Biochem* **10**:338–344.
- Nordøy A, Marchioli R, Arnesen H, and Videbaek J (2001) n-3 polyunsaturated fatty acids and cardiovascular diseases. *Lipids* **36**:S127–S129.
- Rapp JH, Connor WE, Lin DS, and Porter JM (1991) Dietary eicosapentaenoic acid and docosahexaenoic acid from fish oil. Their incorporation into advanced human atherosclerotic plaques. *Arteriosclerosis Thrombosis* **11**:903–911.
- Salem N Jr, Litman B, Kim HY, and Gawrisch K (2001) Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* **36**:945–959.
- Shimokawa H and Vanhoutte PM (1989) Dietary omega 3 fatty acids and endothelium-dependent relaxations in porcine coronary arteries. *Am J Physiol Heart Circ Physiol* **256**:H968–H973.
- Simopoulos AP (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* **21**:495–505.
- Thid D, Benkoski JJ, Svedhem S, Kasemo B, and Gold J (2007) DHA-induced changes of supported lipid membrane morphology. *Langmuir* **23**:5878–5881.
- Toro L, Marijic J, Nishimaru K, Tanaka Y, Song M, and Stefani E (2002) Aging, ion channel expression, and vascular function. *Vascul Pharmacol* **38**(1):73–80.
- Wu KT, Huang CT, Wei J, Tsait LM, Hsu CH, Chen YC, Yangs JM, and Lin CI (2007) Vasodilator action of docosahexaenoic acid (DHA) in human coronary arteries in vitro. *Chin J Physiol* **50**:164–170.
- Ye D, Zhang D, Oltman C, Dellsperger K, Lee HC, and VanRollins M (2002) Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *J Pharmacol Exp Ther* **303**:768–776.
- Yokoshiki H, Sunagawa M, Seki T, and Sperelakis N (1998) ATP-sensitive K⁺ channels in pancreatic, cardiac, and vascular smooth muscle cells. *Am J Physiol Cell Physiol* **274**:C25–C37.

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